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01APR03 E796695-8 D02819
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3.	Full name, address and postcode of the or of each applicant *(underline all surnames)*	Creative Gene Technology Ltd Integrative Cell Biology Laboratory School of Biological and Biomedical Sciences University of Durham, South Road Durham DH1 3LE
	Patents ADP number *(if you know it)*	860199001
	If the applicant is a corporate body, give the country/state of its incorporation	United Kingdom
4.	Title of the invention	
5.	Name of your agent *(if you have one)*	Keith W Nash & Co
	"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*	90-92 Regent Street Cambridge CB2 1DP
	Patents ADP number *(if you know it)*	1206001

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Keith W Nash & Co

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C891.00/S

Title: Improvements in or Relating to Plant Viability

Field of invention

This invention relates inter alia, to a method of killing plant cells, and to compositions for killing plant cells.

Background of invention

List of abbreviations

The following abbreviations are used in this document:

ABC	ATP binding cassette
ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
AMP-PCP	β,γ-methyleneadenosine 5'-triphosphate
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
CHAPS	3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate
DTT	Dithiothreitol
IPG	Immobilised pH gradient
mRNA	messenger RNA
MS	Murashige and Skoog
NTPase	nucleotide triphosphatase
PCR	polymerase chain reaction
RNAi	RNA interference
SDS	sodium dodecylsulphate

ATP is a ubiquitous, energy-rich compound that is found in all cells of free-living organisms. It is found both within organelles, such as mitochondria and chloroplasts, as well as in the cytoplasm. Glycolysis, oxidative phosphorylation and photophosphorylation are some of the cellular biochemical pathways capable of generating ATP. The energy from ATP is used to drive a number of essential biochemical reactions that are fundamental to the survival of cells and whole organisms. The presence of intracellular ATP has been recognised for a long time since its discovery in living cells (Fiske & Subbarow 1929 Science 70, 381-382).

Because of its molecular size and charge, ATP cannot cross the plasma membrane by simple diffusion and, therefore, would not normally be expected to occur extracellularly in the absence of cytolysis. However, two alternative mechanisms by which cells secrete ATP have been discovered. The first is exocytosis, a mechanism predominantly used by (but not exclusive to) nerve terminals where the released ATP functions as a neurotransmitter. The second mechanism utilises ABC transporters directly or by indirect activation of ATP channels. ATP release from animal cells was first reported in 1959 (Holton, 1959, J. Physiol.(London) 145, 494-504).

The presence of extracellular ATP in a plant system was only recently recorded in *Arabidopsis* in 2000 (Thomas et al., 2000 Plant Cell 12, 519-533) and there are no reports of its occurrence in any other plant species. ATP extrusion in *Arabidopsis* is up-regulated in mutants over-expressing an ABC transporter (Thomas et al., 2000), suggesting that *Arabidopsis* employs this mechanism for export. Even though external ATP has only been recorded in *Arabidopsis*, the widespread presence of ABC proteins in plants (both monocots and dicots) suggests that the existence of extracellular ATP may be prevalent in the plant kingdom, though experimental evidence is still lacking. However the role, if any, of extracellular ATP in plants is not known. Despite the existence of extracellular ATP in plants, it has hitherto been regarded as only a by-product of ABC protein activity with no relevance whatsoever to plant growth and development. The single publication on plant release of ATP views this phenomenon as a possible waste of valuable phosphate that the cells rectify by employing extracellular NTPases to cleave the phosphate groups from

the ATP and facilitate their re-absorption. Against this background, there is no reason for the person skilled in the art to conclude that the level of external ATP is vital for the viability of plant cells.

Controlling cell death/viability has long been important in the development of targets for new herbicides. Selective cell viability is also important as a way of altering development of plants by causing death of important tissues and cell lineages. This has application in male-sterility in plants and possibly altering developmental morphology of entire organisms. In addition, delaying cell death can extend the longevity of plants and this can be of commercial importance. For example, delaying flower and leaf abscission by controlling cell death can potentially increase the "shelf-life" of ornamental plants. Moreover, prevention of flower and fruit abscission automatically increases the yield of crop plants. The control of cell viability is also important in disease control. Various treatments have been devised which use specific delivery systems for killing particular cell types. The inventors herein demonstrate that cell death can be mediated by reduction of extracellular ATP levels and/or by preventing its hydrolysis by cellular enzymes. This has utility in developing targets for new herbicides and control of organism development. It will also have application in the development of new pathogen resistance strategies in plants.

All publications mentioned in this specification are specifically incorporated herein by reference.

Summary of Invention

The present invention provides a novel way of killing plant cells and whole plants by, for example, depleting the amount of extracellular NTP (nucleotide triphosphate), especially ATP, available to plant cells. The invention allows one to select/identify targets for generation of new herbicides, novel strategies to control diseases, and the control of cellular or whole plant morphology. It is demonstrated that suspension cultures of *Arabidopsis* and corn (*Zea mays*) are killed when extracellular ATP is removed or

competitively excluded from its binding sites. Two examples are given for the removal of extracellular ATP from cell suspension cultures; (i) incubation with glucose and hexokinase, which utilises ATP to generate glucose-6-phosphate, and (ii) incubation with apyrase, an enzyme that hydrolyses ATP to AMP and inorganic phosphate in a 2-step reaction with ADP as an intermediate product. Addition of AMP-PCP, a non-hydrolysable analogue of ATP, is an example of killing cells by effectively decreasing the level of extracellular ATP available to enzymes by competing for binding the active site and so excluding ATP from participating in essential biochemical processes. The requirement for external ATP is further extended to whole plants by demonstrating that application of any of the 3 treatments used to kill cell suspension cultures results in localised necrotic lesions when applied locally and death of whole leaves when applied to a whole tissue. Mechanisms that result in the lowering of external ATP or rendering it non-available to the plant cell (e.g. prevention of its utilization by cellular enzymes) can thus be used to mediate cell death. This can be done in a variety of different ways, as described more fully below.

Conversely, the inventors have found that in conditions in which extracellular ATP is undesirably depleted or in which undesirable depletion of extracellular ATP is triggered (e.g. by the presence of pathogens), then cell viability can be enhanced or improved by addition of exogenous ATP.

The inventors believe that a similar effect may result following depletion of the extracellular concentration of one or more of the other naturally-occurring nucleotide triphosphates (CTP, GTP, TTP). Whilst it is preferred that the invention concerns alteration of the extracellular concentration of ATP, it is possible that the same or a similar pathway is activated by depletion of the other nucleotide triphosphates (NTP). Thus, the term ATP as used herein is generally to be construed as equally encompassing the other nucleotide triphosphates unless the context dictates otherwise.

Thus, in general terms, the invention provides a method of controlling the viability of a plant cell or cells by contacting the cell or cells with a substance which directly or

indirectly up- or down-regulates a cell death pathway in the cell or cells, which pathway is activatable by depleting the concentration of NTP, especially ATP, in the extracellular environment available to the cell or cells. By depleting extracellular NTP, or triggering such a depletion, the viability of the cell or cells can be abolished whilst, for a cell or cells exposed to extracellular NTP depletion, the viability can be preserved by providing exogenous NTP, especially exogenous ATP.

More specifically, in one aspect the invention provides a method of killing a plant cell by activating a cell death pathway, which pathway is activatable by depletion of extracellular NTP, especially ATP, available to the cell for utilization (e.g. by cellular NTPase enzymes).

In this context "cellular derived NTPase enzymes" is intended to encompass enzymes which are secreted or otherwise exported to the exterior of the cell or are present on or in the cell membrane, and includes multi-activity enzyme complexes which possess an NTPase activity. Note that NTP binding to a particular receptor (rather than its hydrolysis by an NTPase) may be sufficient to modify the viability of the cell.

"Activating" the cell death pathway means triggering the pathway in some manner such that, after activation, the activity of one or (preferably) more enzymes, which catalyse particular reactions in the pathway, is increased. The activity may increase by accumulation of greater amounts of the enzyme/s in question and/or by conversion of the enzyme from a relatively inactive form to a relatively active form (e.g. by dephosphorylation). The pathway may be activated at an upstream end (e.g. by depletion of extracellular NTP, especially ATP) and/or at one or more intermediate points downstream of extracellular NTP depletion.

In classical biochemical terms, a pathway may typically be activated by increasing the concentration of a substrate of the pathway and/or by depleting the effective concentration of the product/s of the pathway.

The plant cell whose viability is to be controlled may be in culture *in vitro* or may be part of a plant or plantlet. Thus, in a particular embodiment the invention provides a method of killing a plant or part thereof, by killing a plurality of the cells within the plant or part thereof to be killed. Those skilled in the art will appreciate that it is not necessary to kill all the cells of a plant or part thereof in order to kill the plant or part thereof (as appropriate) - killing, for example, the majority of the cells will normally render the remainder non-viable in due course.

Equally, whilst activation of the extracellular NTP-depletion mediated cell death pathway can be used to abolish plant cell viability, inhibition of the pathway at one or (preferably) more points will act to preserve viability of plant cells in conditions of extracellular NTP-depletion or other circumstances which would tend to trigger cell death. The inventors can envisage a number of ways of inhibiting the cell death pathway. Typically a pathway may be inhibited or down-regulated by the use of a substance which utilises components of the pathway in such a way as to divert the cell death "signal". The substance may be, for example, a reversible or irreversible inhibitor of one or more enzymes in the pathway. Classically, such inhibitors may be a structural analogue of the enzyme's intended substrate and thereby prevent the enzyme acting on its intended substrate, in a competitive or non-competitive manner.

An alternative approach would be to cause over-expression of polypeptides in the cell death pathway which are altered in some way (e.g. mutated) so as to render them inactive (in the sense of being unable to propagate the "cell death signal" along the pathway), then such inactive polypeptides would compete with the plant cell's intrinsic active proteins and effectively swamp them. Such altered polypeptides could of course be expressed in the plant cell by genetic modification, e.g. introducing a nucleotide sequence into the plant cell which expresses the altered polypeptide at high concentration.

In a further aspect the invention provides a composition for controlling the viability of a plant or plant cell, the composition comprising an active agent which, directly or indirectly, up- or down-regulates in the cell or cells, a cell death pathway, which pathway

is activatable by depletion of extracellular NTP, especially ATP, available for utilization by the cell or cells. The composition will advantageously comprise other constituents conventionally present in herbicidal formulations, and which will be well known to those skilled in the art, such as surfactants and penetration enhancers, (see, for example, Brand & Mueller 2002, Toxicological Sciences 68, 18-23, and references cited therein).

Typically the composition will be made and sold as a concentrate, which must be diluted with water or other diluent before use.

One way of activating the relevant cell death pathway discovered by the inventors is to cause depletion in the extracellular environment of the level of NTP, especially ATP, available for hydrolysis or other utilization by the plant cell. This may be done by actually removing or destroying extracellular NTP and/or may be achieved by otherwise rendering the extracellular NTP present non-available to the cell.

Preferably the method of the invention involves the step of bringing an active agent into contact with the extracellular environment of the cell or cells to be killed, which agent has the effect of hydrolysing extracellular NTP (especially ATP) and/or rendering extracellular NTP (especially ATP) non-available to the plant cell or cells, or otherwise activating the cell death pathway.

Agents which hydrolyse NTP include apyrases, or kinases (preferably in combination with a suitable phosphate group acceptor substrate, e.g. hexose kinase and hexose, especially glucosekinase and glucose).

Agents which render, for example, extracellular ATP non-available to the plant cell include compounds which bind to ATP and prevent its uptake or use by the plant cell. Other compounds which render the extracellular ATP non-available to the plant cell include compounds which are competitors of ATP i.e. substances which will bind (preferably with an affinity equivalent to or greater than that of ATP), to ATP-binding sites on the exterior of the cell. (A competitor which has a lower binding affinity than

ATP may nevertheless be effective if it can be provided at a concentration which effectively swamps any extracellular ATP present.) Such ATP-binding sites will typically be present on kinases or other ATP-hydrolysis-linked enzymes. In particular, the competitor compound may be an analogue of ATP, especially a non-hydrolysable analogue of ATP, such that once the competitor has occupied the ATP-binding site on the exterior of the cell it will essentially prevent ATP being subsequently bound. Examples of non-hydrolysable analogues of ATP include AMP-PCP. Non-hydrolysable analogues of other NTPs may also be useful.

Those skilled in the art will appreciate that the cell death pathway which is activatable by the depletion of extracellular NTP available for utilization may equally be activated (or inhibited) at a point downstream of NTP depletion. For example, the inventors provide evidence (below) that extracellular ATP depletion results in the dephosphorylation of several plant cell polypeptides. It may be hypothesised that one or more of these polypeptides must be in phosphorylated form in order to retain a desirable biological activity, such that dephosphorylation will eventually kill the plant cell. Detailed genomic and proteomic studies, along the lines of those proposed below in the Examples, and within the capability of those of normal skill in the art, will reveal the changes which take place in the cell at the nucleic acid and polypeptide level when the cell death pathway is activated, and thereby reveal other methods of and agents for activating the pathway.

The composition of the invention may be selectively applied e.g. by injection or surface application to particular parts of plants to cause cell death restricted to desired portions. Alternatively, substantially all of the plant may be exposed to the agent (e.g. by spraying the composition onto the plant), to cause the death of the whole plant.

The inventors have found that exposure of plant cells to bacterial pathogens (as exemplified by *Ps. syringae*) or fungal pathogens (as represented by *Fusarium* elicitor substance), can cause cell death by a mechanism which involves depletion of extracellular NTP, and that the viability of plant cells exposed to these agents can be restored to near normal levels by causing an increase in NTP concentration in the extracellular environment of the cells.

Thus, in a particular further embodiment within the overall concept of the invention, there is provided a method of preserving the viability of a plant cell or cells exposed to a viability-threatening depletion of extracellular NTP (whether triggered by presence of pathogens or other causes), the method comprising the step of administering a viability-preserving substance which has the effect of increasing the extracellular NTP concentration or otherwise inhibiting the cell death pathway which has been activated (e.g. by a pathogen). Where the method is performed *in vitro*, the viability-preserving substance may simply be introduced into the culture medium. Where the method is performed *in planta*, the viability-preserving substance may conveniently be introduced into the plant by spraying onto the surface thereof, or by application in solution to the soil or other water-source of the plant, or less preferably by direct injection into the plant. Most simply, the viability-preserving substance comprises NTP (especially ATP) but may be any substance which has, as a result of interaction with one or more other substances present in the plant cell and/or in the extracellular environment, the effect of augmenting the extracellular NTP (especially ATP) concentration or otherwise inhibiting the cell death pathway. This method of the invention may find particular usefulness in protecting a plant against attack by pathogens.

It might also be possible to produce new plant varieties by conventional breeding or by genetic manipulation/modification, for example, introducing a gene or genes encoding for polypeptide(s) which inhibit or counteract extracellular NTP (especially ATP) depletion caused or triggered by pathogens, or otherwise inhibit the cell death pathway, and thereby render the new variety resistant to cell death e.g. caused or triggered by certain pathogens. For instance, the viability of a plant cell could be preserved by introducing nucleotide sequences to encode the components of a biochemical pathway which result in elevated levels of extracellular NTP (especially ATP) either constitutively or specifically in response to an event (such as attack by a pathogen) which tends to deplete extracellular NTP (especially ATP). Such sequences and/or the polypeptides encoded thereby can be regarded as a viability-preserving substance.

Still other embodiments of the invention may be envisaged. For example, it can be hypothesised that the presence of extracellular NTP (especially ATP) is required in order to transmit a "stay alive" signal. This might be propagated, for example, by polypeptides or other substances, probably in a phosphorylated form. Thus over-expression of polypeptides involved in propagating the "stay alive" signal may preserve viability of the plant cell, even in conditions (such as pathogen attack) in which extracellular NTP (especially ATP) is depleted.

The invention will now be further described by way of illustrative example and with reference to the accompanying drawings, in which:

Figure 1 is a bar chart showing ATP level (expressed as a % of levels in control cultures) after various durations (in hours) of ATP depletion treatments. The error bars show the standard deviation;

Figure 2A and 2B are photomicrographs showing (A) live cells of *Arabidopsis thaliana* treated with O units/ml apyrase and (B) dead cells of *Arabidopsis thaliana* treated with 100 units/ml apyrase;

Figures 3A-C and 4A-C are bar charts showing cell viability (expressed as packed cell volume %) for plant cell suspension cultures subjected to control or various experimental treatments. The error bars show the standard deviation;

Figures 5A, B, 6A-C and 7A-D are photographs of plant leaves showing the effects of control or experimental treatments on various plants;

Figures 8A-F, 9A-C and 10A, B are photographs of plants showing the effects of control or experimental treatments on various plants;

Figures 11A-D are photographs showing the results of 2D-gel electrophoresis analysis of extracellular proteins of *A. thaliana* cultures;

Figure 12A is a graph of % extracellular ATP level against time (in hours); and

Figure 12B is a bar chart showing cell viability (arbitary relative units) for *Arabidopsis* cultures 24 hours after various treatments.

EXAMPLES

Example 1. Treatment of *Arabidopsis thaliana* cell suspension cultures with apyrase or hexokinase/glucose removes extracellular ATP.

A suspension of *Arabidopsis thaliana* cells was grown in MS medium (Murashige & Skoog, 1962 Physiol. Plant. 15, 473-497) with minimal vitamins and containing 3% (w/v) sucrose, 0.5mg/L kinetin, and 0.5mg/L 1-naphthalene acetic acid, and adjusted to pH 5.7 with NaOH/HCl. All the medium components were purchased from Sigma Chemical Company (Poole, UK). The culture was propagated by weekly sub-culturing of 7 day old inoculum into fresh medium (10-fold dilution) and incubating on a rotary platform (125 r.p.m) at 25°C in complete darkness. Such cells are viable for many days after transferring to fresh growth medium. Cells were normally grown as 100mL cultures in 250mL glass Erlenmeyer flasks, but all treatments were performed on 1.5mL or 10mL aliquots in sterile plastic vials of 3.5cm diameter (Bibby Sterilin Ltd., Stone, UK). Cell cultures were used for treatments 3 days after inoculating fresh medium.

A final concentration of 100mM glucose (filter sterilized) was added to a 3 days old cell suspension culture that was then divided into 10mL aliquots. The aliquots were treated with a final concentration of 100 units/mL apyrase or 200 units/mL hexokinase. Both apyrase and hexokinase (Sigma Chemical Co.) were dissolved in deionised water and filter-sterilised using 0.2μm filters. Control cultures were treated with an equivalent volume (2ml) of sterile deionised water. The cultures were incubated for a total of 25 hours, with 500μL aliquots of culture medium being withdrawn for ATP assays at 0, 8, 12, and 25 hours after treatment. The 500μL aliquots were mixed with 10μL of 50% (w/v) trichloroacetic acid containing 0.0005% (w/v) xylene cyanole FF and immediately

frozen in liquid nitrogen. These samples were then thawed and the precipitated protein pelleted and discarded. For ATP assay, 5μL of the supernatant were mixed with 95μL of 100mM Tris-acetate buffer (pH 7.8) in wells of non-transparent 96-well microtitre plates. The assay was performed on duplicate samples by adding 30μL of a luciferin/luciferase mix reagent (Promega, Southampton, UK) followed by a reading delay of 0.3 seconds and an integration time of 2 seconds. The 30μL of luciferin/luciferase mix were applied via an automatic reagent feeding line fitted to the luminometer (model Anthos Lucy 1; Labtech International Ltd., Ringmer, UK). Water and fresh growth medium were used as blanks.

The amount of extracellular ATP in the treated cultures was expressed as a percentage of extracellular ATP in the control cultures at each time point. Figure 1 is a time-course of extracellular ATP levels in *Arabidopsis* cell suspension cultures treated with apyrase (gray blocks) and hexokinase (spotted blocks) as a percentage of the extracellular ATP in control cultures. Error bars represent the standard deviation. The figure shows that both apyrase and hexokinase had reduced the amount of extracellular ATP levels below 5% of the amount in the control cultures within 25 hours of commencing treatment. Hexokinase phosphorylates glucose to glucose-6-phosphate by transferring a phosphate group from ATP, and thus producing ADP. Apyrase dephosphorylates ATP to ADP and ADP to AMP. These reactions consume ATP and account for the observed reduction of extracellular ATP attending the treatment of cell cultures with both enzyme systems. However, hexokinase was more rapid in reducing the level of extracellular ATP in this system than apyrase, as it reduced extracellular ATP to levels less than 5% within 8 hours of treatment. This is because the intermediate product of ATP catabolism by apyrase is also a substrate for this enzyme, hence the rate of ATP dephosphorylation decreases as more ADP is produced. These results demonstrated that apyrase and hexokinase activities are sufficient to effectively reduce the amount of extracellular ATP in a plant cell suspension culture system.

Example 2. Removal of external ATP by treatment of *A. thaliana* cell suspension cultures with apyrase results in cell death.

A. thaliana cell cultures were grown as described in example 1. Aliquots of the cell suspension (1.5mL) were treated with a final concentration of 0, 20, 50, or 100 units/mL of apyrase. The cultures were incubated for 3 days and the apyrase-treated cultures showed a significant frequency of cell death. The dead cells had become buoyant and adhered to the walls of the vials, forming a ring just above the edge of the swirling medium. The ring of cells was sometimes dislodged and fell into the medium, resulting in apyrase-treated cultures having flakes of dead cells at the bottom of the vials.

The inventors decided to use viability staining to confirm cell death in these cell cultures. To achieve this, 200µL aliquots were removed from the cultures and the cells resuspended in 0.2M $CaCl_2$ after removal of the growth medium. The aliquots were doubly-stained by incubating for 5 minutes with a final concentration of 25µg/mL fluorescein diacetate [0.5% (w/v) stock solution in acetone] and 50µg/mL propidium iodide [10mg/mL stock solution in phosphate-buffered saline pH 7.4]. Microscopic examination under UV light revealed live cells, which were emitting green fluorescence (Figure 2A). The viable cells had also excluded propidium iodide, which is non-permeative and can only cross membranes of dead or dying cells. Dead cells had taken up propidium iodide, which binds to DNA, resulting in nuclei emitting a very intense red fluorescence (Figure 2B). The inventors observed that a significantly high proportion of cells in the apyrase-treated cell cultures was dead and it was confirmed that the flakes of cells found at the bottom of these cultures were indeed dead.

To quantify the effect of apyrase on cell viability, the ability of the cells to grow and multiply subsequent to treatment was measured after diluting the cultures by over 30 times in fresh medium not containing any additives. This was achieved by transferring the cells treated for 3 days to 50mL of fresh growth medium and allowing them to grow for a further 4 days. At the end of this period, triplicate 1mL aliquots were sampled from the cultures and the volume of the cells was measured and expressed as a percentage of the culture volume. The method used to determine the packed cell volume was as follows: 1 mL cell culture aliquots were placed in 1.5 mL microfuge tubes and the cells gently compacted by centrifuging (1000 rpm., 10 minutes) in a swing-out rotor (Grundrotor

11030; Sigma laborzentrifugen GmbH, Oestrode, Germany). The level of the cells was marked on the wall of the tube and the volume of cells determined by measuring the volume of water needed to fill the tube to the marked level. The packed cell volume was expressed as the volume occupied by cells as a percentage of 1000 μL.

Figure 3A shows the dose-response of *Arabidopsis* cells to treatment with apyrase at 0, 20, 50 or 100 units/ml. Figure 3B shows the results obtained when using native or boiled apyrase and glucose (100mM) in combination with native or boiled hexokinase (apyrase at 50 units/ml, hexokinase at 200 units/ml). Figure 3C shows the effects of treating the cells with either ATP (1mM) or with glucose-6-phosphate (100mM), AMP(1mM) or ADP (1mM). In each case the error bars represent the standard deviation.

As apparent from Figure 3, increasing apyrase concentration caused a progressive decrease in cell viability with over 80% loss in viability at 100 units/mL apyrase. When apyrase was denatured by boiling for 5 minutes prior to treating the suspension cultures, the treatment did not result in cell death (Fig. 3B) revealing that the cell death resulting from treating cells with native apyrase requires the enzyme to be active. Treatment of cell cultures with ADP or AMP, products of the reaction catalysed by apyrase, did not result in any change in cell viability (Fig. 3C). Since apyrase is cell-impermeative and, therefore, a mechanism to selectively destroy extracellular ATP, these results demonstrate that deprivation of extracellular ATP triggers a cell death response.

Example 3. Removal of external ATP by treatment of *A. thaliana* cell suspension cultures with hexokinase and glucose results in cell death.

Arabidopsis cell cultures were grown and treated as described in example 2 and the glucose plus hexokinase treatment was used as the extracellular ATP removal system. The cultures were treated with a combination of 100mM glucose and 0, 20, 50, 100, or 200 units/mL hexokinase. The results are shown in Figure 4A. As apparent from the Figure, cell viability was progressively lost with increasing hexokinase concentration and 200 units/mL hexokinase and 100mM glucose treatment was attended by an over 80% loss of

viability. Although glucose can freely diffuse into cells, hexokinase is cell-impermeative and remains in the external medium, and its addition to cell cultures results in a targeted removal of extracellular ATP. Hexokinase that had been denatured by boiling for 5 minutes before addition to cell cultures did not cause cell death (Fig. 3B), demonstrating the absolute requirement for a native enzyme for cell death to ensue. Treatment of cells with ADP or glucose-6-phosphate, products of the reaction catalysed by hexokinase, did not affect cell viability (Fig. 3C). This result shows that removal of extracellular ATP compromises viability of *Arabidopsis* cells in suspension culture.

This finding was specific to plant cells: comparable experiments with combinations of glucose/hexokinase had no significant effect on the viability of the bacterium *E. coli* or the yeast *S. cerevisiae* (data omitted for brevity).

Example 4. Treatment of *A. thaliana* cell suspension cultures with a non-hydrolysable ATP analogue, AMP-PCP, results in cell death.

As cell death could be triggered by the removal of extracellular ATP, the inventors predicted that a compound that could compete with ATP metabolically or in signaling processes and is not hydrolysable, would also cause cell death. This was tested by the application of a non-hydrolysable analogue of ATP, β,γ-methyleneadenosine 5'-triphosphate (AMP-PCP), to *Arabidopsis* suspension cells. Because of its molecular size and charge, AMP-PCP cannot diffuse into cells and, therefore, selectively interferes with processes that utilise only extracellular ATP. The cell cultures were treated with 0, 0.5, 1.0, and 1.5mM AMP-PCP in an experiment conducted as described in example 2. A 45.5mM stock solution of AMP-PCP that had been adjusted to pH 6.5 using KOH was used. The dose-response of these cells to the ATP analogue is shown in Figure 4B. Progressively increasing the concentration of AMP-PCP was accompanied by a loss in cell viability that reflected the occurrence of cell death due to a competitive exclusion of ATP from binding sites by the analogue. Treatment of cells with 1mM ATP did not cause death of treated cells (Fig. 3C). These results confirm that extracellular ATP is required for the

viability of suspension cells and that non-hydrolysable analogues of ATP can be used to kill cells.

Example 5. Treatment of *Zea mays* suspension cells with apyrase, hexokinase/glucose, or a non-hydrolysable ATP analogue results in cell death.

Black Mexican sweet corn cells were grown in MS medium with 2% (w/v) sucrose and 2mg/L 2,4-dichlorophenoxyacetic acid, and adjusted to pH 5.7 with NaOH and HCl. The cultures were maintained by weekly sub-culturing 7 day old inoculum into fresh medium (10-fold dilution). Treatment of the cell cultures was performed 3 days after transferring to the fresh growth medium. A final concentration of 100mM glucose was added to the culture before aliquoting 1.5mL each into the plastic vials for treatment. The cell cultures were treated as described in example 2 using the following final concentrations; 1mM ATP, 1mM AMP-PCP, 100 units/mL apyrase, and 200 units/mL hexokinase. The control was treated with an equivalent volume of sterile deionised water alone. The response of corn cells to these treatments was similar to that of *Arabidopsis* cultures as shown in Figure 4C. As expected, ATP did not affect the viability of the cells, but AMP-PCP, apyrase, and hexokinase/glucose caused significant cell death levels equivalent to those caused in the *Arabidopsis* cultures. This demonstrated that extracellular ATP is indispensable for the viability of a monocot (corn) cell system, as is the case in the dicot *Arabidopsis*.

Example 6. Exogenous application of extracellular ATP depletion systems to areas of a whole plant causes cell death and development of necrotic lesions or death of entire tissues.

The effect of local application of extracellular ATP removal systems and a non-hydrolysable ATP analogue was evaluated on whole plants. Tobacco (*Nicotiana tabacum*), *Arabidopsis*, and bean (*Phaseolus vulgaris*) plants were sown in soil and raised in a growth cabinet with a 16-hour photoperiod at 20°C and 8 hours of darkness at 15°C. The relative

humidity was maintained at 60% and the photon flux density was 250$\mu molm^{-2}s^{-1}$. The plants were used for treatment with the extracellular ATP removal systems when they were 5-6 weeks old. Only the cotyledons of bean plants were treated. The abaxial surface of a small zone of leaf tissue was treated by infiltrating the apoplast with the solution using a syringe and hypodermic needle. The three test solutions had the following concentrations of either: 0.5 units/μL apyrase, 1.85units/μL hexokinase plus 100mM glucose, or 1-5mM AMP-PCP (pH 6.5). All three systems resulted in the development of necrotic lesions in the area where the application was made within 2 days of treatment (Fig. 5A, 6-7). Similar applications (control) without the active ingredients did not result in necrotic lesions.

In Figure 5A, the arrow indicates the needle prick on the control leaf (top panel, (i)) or the localised necrotic lesions that develop after the application of one of the various extracellular depletion systems (ii = 5mM AMP-PCP; iii = apyrase; iv = hexokinase + glucose).

Treatment with ATP (pH 6.5) or individual products from the reactions catalysed by apyrase and hexokinase did not result in cell death (e.g., Fig. 6A).

Figure 6 shows the results obtained following treatment of tobacco leaves with various solutions. Panel A shows the absence of a reaction except for a needle prick wound after treating the leaf with water (1), 6mg/mL BSA (2), 1mM AMP plus 1mM ADP (3), and a combination of 50mM glucose/50mM glucose 6-phosphate/1mM ADP (4). Panel B shows the development of localised necrotic lesions after treatment with apyrase (5) and hexokinase plus glucose (6). In panel C, infiltration of 1mM AMP-PCP caused localised tissue death (7) whereas treatment with 1mM ATP did not (8).

Figure 7 illustrates the development of necrotic lesions (arrowed) on bean leaves after treatment with apyrase (C) or hexokinase (D) and the lack of reaction (needle prick sites arrowed) of tissues treated with water (A) or 100mM glucose (B).

Localised treatment was used to show the contrast with adjacent living tissue. It is therefore clear that the cell death associated with the removal of extracellular ATP is not restricted to cells in suspension culture, but also applies to whole plants.

In order to demonstrate that the effect of extracellular ATP removal could be much more widespread than a localised necrotic lesion, the same depletion systems were applied to an entire leaf of the 3 plant species used above. The concentrations used were the same as defined above unless stated otherwise. This treatment resulted in the collapse of the treated tissues within 24 hours of treatment and then death of the entire leaf, as apparent in Figures 5B (panels ii-iv), and Figures 8-10.

In Figures 5B, panels ii-iv, the death of the entire leaf is apparent, whilst the control leaf (panel B(i)) remains healthy.

Figure 8 shows the effect of treating an entire leaf (arrowed) of a tobacco plant with one of various solutions: water (panel A), glucose (panel C) and 5mM ATP (panel E) had no adverse effect. Leaves treated with apyrase (panel B), hexokinase/glucose (panel D) or 5mM AMP-PCP (panel F) collapsed 24 hours after treatment (as shown in the Figure) and died completely within a short time.

Similar results (shown in Figure 9) were obtained with bean plants. Figure 9A shows a healthy untreated control plant. Figure 9B shows a plant with one control leaf treated with water (black arrowhead) and one leaf (white arrowhead) treated with apyrase. Likewise, a control leaf is denoted by a black arrowhead in Figure 9C, whilst the white arrowhead indicates a leaf treated with hexokinase/glucose.

Figure 10B shows a tobacco plant treated with 5mM AMP-PCP. After 4 days, not only had the treated leaf died (white arrowhead), but so too had portions of some upper, untreated leaves, indicating that the AMP-PCP had become systemic. The control plant (Figure 10A) treated with an equivalent amount of ATP remained entirely healthy.

Example 6B

Cell death is an essential part of plant development that is regulated by a programmed genetic template which may affect single cells, particular cell layers, or entire organs (Fukuda, 1997 Plant Cell 9, 1147-1156; Groover *et al*, 1997 Protoplasma 196, 197-211; Buchanan-Wollaston, 1997 J. Exp. Bot. 48, 181-199).

The inventors decided to investigate whether pathogen-induced hypersensitive cell death (Lam *et al*, 2001 Nature 411, 848-853) is mediated via hydrolysis of extracellular ATP. Treatment of plant cell cultures with an avirulent pathogen or pathogen-derived elicitors induces the hypersensitive cell death response (Levine *et al*, 1994 Cell 79, 583-593). The inventors reasoned that if this hypersensitive response were mediated via a transitory or sustained removal of extracellular ATP, then it might be abrogated by addition of excess exogenous ATP concomitant with, or shortly after, treatment.

Fusarium moniliforme elicitor prepared as described before (Raventos *et al*, 1995 Plant Journal 7, 147-155) was used in treatments of *Arabidopsis* cell cultures at a final concentration of 100 µg/ml. *Pseudomonas syringae* pv. *tomato* DC3000 strain, possessing the avirulence gene *avrRpm* 1, was grown in standard Luria Bertani medium (Sambrook *et al* 1989 Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York) with 50 µg/ml kanamycin A. Overnight bacterial cultures were harvested (1000 xg for 10 min) and resuspended in water and used to inoculate *Arabidopsis* cell cultures at a final bacterial density of 8.8×10^7 cfu/ml. A concentration of 1 mM ATP was used to investigate the effect of ATP on elicitor- or bacteria-induced death in *Arabidopsis* cell cultures.

The MTT assay (Watts *et al*, 1989 Int. J. Radiat. Oncol. Biol. Phys. 16, 939-942) was used to obtain a quantitative measure of plant cell viability 24 hours after the various treatments of the *Arabidopsis* culture.

The results of these experiments are shown in Figures 12A, B. Figure 12A is a graph of extracellular ATP (as a percentage of that present in control cultures without *Ps. syringae*) against time (in hours). The level of extracellular ATP was measured using the luciferase-

luciferin method and the ENLITEN^{RTM} kit (Promega, Southampton, UK) according to the manufacturer's instrutions.

Figure 12A shows a transient depletion of extracellular ATP peaking about 6 hours after inoculation of the *Arabidopsis* cultures with an avirulent strain of *Ps. syringae* pv. *tomato*.

Figure 12B shows the results of experiments in which *Arabidopsis* cultures were treated with *Ps. syringae* or with *Fusarium* elicitor, alone or with added exogenous ATP. The error bars represent the standard deviation from the mean (n=3). The presence of either *Ps. syringae* or the *Fusarium* elicitor caused a significant reduction in cell viability, which could be substantially negated by the inclusion of exogenous ATP in the cultures at 1mM.

However, rescuing of cells from death by ATP was not via an antioxidant mechanism since ATP failed to prevent H_2O_2 accumulation (data omitted for brevity). This demonstrates that exogenous ATP does not indiscriminately block elicitor effects, but specifically inhibits cell death. Overall, these results are consistent with the conclusion that the cell death pathway mediated by hydrolysis of extracellular ATP is employed in nature during activation of the pathogen-induced hypersensitive response.

The results allow a rationalisation of extracellular ATP, cell death, and the response of plant cells to pathogen elicitors. Elicitors clearly affect cell viability (Levine *et al*, Cell 79, 583-593) and have been reported to activate membrane- and cell wall-bound ATPase activity (Kiba *et al*, 1995 Plant Cell Physiol. 36, 809-817). In plants, extracellular ATP hydrolysis is performed by ecto-apyrases (Komoszynski & Wojtczak, 1996 Biochim. & Biophys. Acta 1310, 233-241; Handa & Guidotti, 1996 Biophys. Res. Commun. 218, 916-923) and cell wall-bound ATPases (Kivilaan *et al*, 1961 Plant Physiol. 36, 605-610; Shiraishi *et al*, 1991 Plant Cell Physiol. 32, 1067-1075). Additionally, a glycopeptide secreted by *Mycosphaerella pinodes* to suppress the induction of pea defence responses also inhibits extracellular ATPase activity (Kiba *et al*, 1995 cited above). Overall, our results reveal a fundamental difference between the effects of extracellular ATP in plants and animals; while extracellular ATP appears to be a crucial requirement for plant cell viability, it is a cytotoxic factor in particular animal cells expressing $P2X_7$ purinoceptors (Ferrari *et al*, 1997 J. Cell.

Biol. 139, 1635-1643; von Albertini *et al*, 1998 Biochem. & Biophys. Res. Commun. 248, 822-829). These findings suggest that the defensive programmed cell death invoked by resistant plants in response to pathogen attack may well be mediated via alteration of the levels of extracellular ATP. This being the case, it should allow transformation of susceptible plant species to become resistant to specific, economically important pathogens by "installing" an extracellular ATP hydrolysis system that can be triggered by presence of the pathogen.

Example 7. Depletion of extracellular ATP alters the state of protein phosphorylation.

The ability of a non-hydrolysable analogue of ATP to mimic the effects of external ATP depletion systems given in examples 2-3 suggested that extracellular ATP hydrolysis is required for maintenance of cell viability. The inventors predicted that removal of external ATP would be attended by changes in the phosphorylation status of some cellular derived proteins. In order to examine this, 30mL of *Arabidopsis* cell cultures containing freshly added 100mM glucose were treated by adding a sealed dialysis tube containing either 6000 units of hexokinase or water. The dialysis membrane had a molecular weight cut-off between 6- and 8 kDa, which confined hexokinase within the dialysis bag. Six hours later, the culture medium was separated from the cells by filtration through 2 layers of Miracloth and clarified by a 15-minute centrifugation at 3000 x *g*. The culture medium proteins were precipitated by incubating at –20°C in 80% acetone for 12 hours. Centrifuging for 10 minutes at 10,000 x *g* pelleted the protein precipitates. The pellets were washed 3 times with 80% acetone and resuspended in a urea buffer (9M urea, 2M thiourea, 4% CHAPS, 1% DTT, 1% IPG buffer 4-7).

Aliquots containing 100µg of protein were loaded into 7cm IPGphor 4-7 gel strips (Amersham Biosciences, Amersham, UK) by the in-gel rehydration technique (Berkelman & Stenstedt 1998 2-D electrophoresis: Using immobilised pH gradients – Principles and Methods. Amersham Pharmacia Biotech, Buckinghamshire, UK). The proteins were separated by 2-dimensional SDS-polyacrylamide gel electrophoresis using standard procedures (Chivasa et al., 2002 Electrophoresis 23, 1754-1765). One set of gels was stained with coomassie and the other was blotted onto nitrocellulose membranes and probed

with a phosphotyrosine-specific antibody (Amersham Biosciences) using standard procedures (e.g., Chivasa et al., cited above).

As the inventors had predicted, it was observed that treatment of cell cultures with glucose/hexokinase alters protein phosphorylation. The results are shown in Figure 11. The top panels show coomassie brilliant blue-stained gel sections of protein samples from glucose-treated (**A**) and glucose/hexokinase-treated (**B**) cell cultures. Bottom panels are western blots of similar gel sections that were immunoprobed with anti-phosphotyrosine serum. Panel **C** is glucose-treated and **D** is glucose/hexokinase-treated. In Figure 11 (**A** and **B**), arrow heads indicate three secreted proteins whose abundance increased slightly in response to treatment with the glucose/hexokinase system as revealed by Coomassie blue staining. Western blots (Fig. 11, **C** and **D**) showed that these proteins contained phosphorylated tyrosine residue(s) and that treatment with glucose/hexokinase resulted in their apparent dephosphorylation. Protein spot 1 is completely dephosphorylated while the signal from spots 2 and 3 is significantly diminished but not totally abolished (Fig. 11**D**). This could arise from the sequestration of extracellular ATP that becomes unavailable to ecto-kinases responsible for phosphorylating the proteins. If ecto-phosphatases and ecto-kinases maintain the balance of phosphorylation of these proteins, then depletion of extracellular ATP by treatment tips the balance in favour of dephosphorylation. Without wishing to be bound by any particular theory, it is possible that changes in protein phosphorylation such as these could be involved in signaling cascades that give rise to cell death. Such phosphorylation changes could be targets for development of new herbicides.

Discussion and conclusions

The present inventors have noted that ATP can be removed from the external medium of cultured cells of both monocot and dicot plant species either by enzymatic cleavage of the gamma and beta phosphate groups with apyrase or by transfer of the gamma phosphate to glucose by hexokinase. ATP removal by either system results in cell death. Quantitative cell death measurements were obtained by determining the ability of the cultures to grow and multiply post-treatment in a volume of fresh growth medium sufficient to dilute the

extracellular ATP removal system by over 30 times. Taken in isolation, this indirect method may also inadvertently measure an arrest of cell division in addition to cell death. However, it was also confirmed that the treatments to remove extracellular ATP cause cell death by viability staining of cell suspension cultures and by demonstrating death of treated, fully expanded plant leaf tissues.

Whilst, the mechanism of cell death is not yet absolutely clear, it is clear that: removal of external ATP results in cell death; mechanisms that override external ATP binding to its target(s) also result in cell death; the level of external protein phosphorylation alters following removal of external ATP and this may also be linked to death; any pathway involved down stream of the "message" resulting in cell death from the above will also result in cell death.

Those skilled in the art could use this basic experimental system to identify changes that occur at the metabolite, mRNA and/or protein level in plants, following such treatments. The skill bases for pursuing such studies are already in place and have been demonstrated (e.g., Ficarro et al., 2002 Nature Biotechnology 20, 301-305; Ideker et al., 2001 Science 292, 929-934). Proteomic analyses using either metabolic labeling, 1- and 2-dimensional gel electrophoresis, and protein identification techniques such as peptide mass fingerprinting, amino acid sequencing and immunology will allow for the identification of such proteins. Similarly, techniques for mRNA abundance determination will allow for the identification of potential gene candidates. Such techniques are readily available and include DNA chip technology, PCR based techniques, differential expression and Northern blot analyses. High throughput metabolic technologies are available to investigate changes in plant metabolites and these could be used to identify a potential block in metabolic pathways that are the result of this special form of "cell death". Once the components have been identified, confirmation of their utility can be realised by gene knockout or gene silencing technologies or by the use of the proteins identified to develop specific inhibitors that will result in cell death. In an analogous fashion, components in the signal transduction pathway could be identified by the development of genetic screens based on lack of responsiveness to the extracellular ATP deprivation death pathway. A detailed description of how the person skilled in the art could identify potential targets for novel herbicidal compositions, and to use

this information in turn to screen for suitable potentially herbicidal agents, is set out below in Example 8.

Example 8

The identification of components in the NTP/ATP-depletion pathway can be achieved by a number of new technologies based on nucleic acid and protein technologies. Once these candidates are identified the corresponding protein can be used as the basis of selecting compounds which will bind to it, either covalently or non-covalently, and developed into specific inhibitors. The essentiality of these proteins for life can be tested by a variety of technologies including antisense, RNAi and identification of appropriate gene-disrupted lines [e.g. lines which are perhaps T-DNA tagged]. The following are given by way of example but are not the only methods of obtaining the identity of the target proteins, mRNA, cDNA and genes, and any of these methods may of course be performed in parallel to obtain confirmatory data.

Example 8A – Use of protein technology to identify targets.

The key to this approach is to identify components which change in quantity or some other discernible characteristic following NTP/ATP-depletion or rescue from it.

Arabidopsis cells, in culture, are treated with appropriate NTP/ATP-depletion conditions and at set points in time following NTP/ATP removal changes in the protein profiles are monitored. A comparison is made between the protein profile of treated and control cells. The cells are harvested at various time points, up to 24 hrs following the treatment, and protein extracts obtained following cellular disruption. Disruption is achieved by use of a French Press as described in Chivasa *et al* [Electrophoresis 23:1754-1765 [2002]] or by other mechanisms, including the use of glass beads – which is equally effective.

The samples are separated into a number of fractions to lower the complexity of the proteins in the sample and achieve greater resolution. These fractions are (i) total cell homogenate, (ii) cell wall fraction, (iii) soluble supernatant protein fraction following centrifugation at

120,000 x *g* for 60 min and (iv) the microsomal pellet fraction from (iii). These samples are analysed by both 1D SDS-PAGE and 2D-gel electrophoresis. Samples are stained with one of a number of dyes to vizualise the proteins, including commassie brilliant blue, silver and Sypro Ruby red. The samples are imaged following eletrophoresis and staining using a ProXpressProteomics Imaging System [Perkin Elmer Life Sciences], quantified using image analysis software and the bands and spots which show changes identified. Spots and bands are picked using the Genomic Solutions ProPic work station and digested to produce peptides using an automated Genomic Solutions Progest robot. Following digestion, and sample work up, as described in Maltman *et al* (Electrophoresis 23:626-639, 2002) the proteins are identified by MALDI-TOF peptide mass fingerprinting, using a PE-Biosystems Voyager-DE STR mass spectrometer and searching of data bases using the PE Biosystems PS1 software.

Alternatively or additionally peptide sequencing may be performed with a Q-Star triple Quad mass spectrometer and the amino acid sequence of the peptides used to determine the protein from which it originates. As an alternative to the post-gel staining described above the gels could be prestained with two different Cy dyes and the differences identified using 2D-DIGE technology as described by Tong *et al* (Proteomics 1:377-396, 2001) and Orange P (Amersham Life Sciences News 5:2000). Following this the differentially expressed spots can be identified and the proteins identified as described above. Alternative technologies could be used to look at differentially expressed proteins by means other than gel separation techniques, such as Isotope Coded Affinity Tag techniques described in Ideker *et al* (Science 292:929-933, 2001) and Gygi *et al* (Nature Biotechnology 17:994-996, 1999) and mass spectroscopic identification.

Example 8B

Protein fractions could be prepared in the same way as in Example 8A above but in order to identify new proteins which were synthesised in response to extracellular ATP depletion (or rescue therefrom) the cells would be labelled with 35S methionine or other radioactive amino acids. In this way following sample fractionation, as above, and fluorography following electrophoresis, candidate proteins which were newly synthesised and differed

between control and test samples can be identified. This has the advantage that new proteins which are synthesized in response to the altered conditions of the cell can be selectively identified within a background of other cellular proteins.

Example 8C

Arabidopsis cell suspensions are treated with water [sample a] or *Fusarium* elicitor [sample b] and a third reaction is performed using the same elicitor but in the presence of 1mM ATP [sample c]. The three samples are compared using the variety of techniques described in Examples 8A and 8B, revealing specific changes attributable to activation of the elicitor mediated cell death pathway which could be reversed by addition of ATP.

Example 8D – Use of nucleic acid technology to identify candidates in the cell death pathway

Experiments are performed using ATP-depletion with controls as described in examples 8A-C above. At set time points following treatment, from 5 min to 2 hrs, mRNA is extracted from each of the samples. RNA may be extracted from *Arabidopsis* cell suspension cultures using a modification of a published method (Fuerst *et al* 1996 Plant Physiol. 112, 1023-1033). Essentially, cells are harvested by vacuum filtration through cellulose filters, flash-frozen in liquid nitrogen and RNA is extracted using the QIAGEN RNeasy kit, followed by washing in 3M sodium acetate to remove contaminants. Fifty microgrammes of total RNA are used to make cDNA by reverse transcription using standard protocols (e.g. Sambrook *et al*, Molecular Cloning. A laboratory Manual. Second edition, Cold Spring Harbor Laboratory Press) from each of the samples in a particular treatment. cDNA samples will be analysed for transcriptional profiles using the Affymetrix oligonucleotide GeneChip (Harmer *et al*, 2000 Science 290, 2110-2113), which contains representation of ca. 24,000 *Arabidopsis* genes. Double-stranded cDNA is transcribed to form biotin-labelled cRNA, which is fragmented by metal hydrolysis prior to hybridization to the GeneChip. Genes which are differentially expressed over 2-fold are identified following cluster analyses. The alteration in expression of these genes is confirmed using both real-time PCR and Northern analyses. RT-PCR and northern analysis on *Arabidopsis* RNA samples may be carried out

using published protocols (e.g. Casson *et al*, 2002 Plant Cell 14, 1705-1721). In this way genes, the expression of which is altered following ATP depletion, could be identified and, in particular, early and late responding genes could be identified.

Example 8E – Use of fungal elicitors

Experiments are performed using *Fusarium* elicitor, with and without 1mM ATP addition, using *Arabidopsis* suspension cultures as described in Example 8C above. At set time points following treatment, from 5 min to 2 hrs, mRNA is extracted from each of the samples. Genes with an altered expression in response to *Fusarium* elicitor treatment, which alteration is reversible by ATP addition, may be identified using protocols described above in Example 8D. These genes represent potential targets for new herbicides.

Example 8F – Development of screens for potential herbicides

A suitable screening procedure will involve a number of steps:

1. Verification that the target gene is essential to life. This may be done, for example, by means of RNAi; looking for T-DNA tagged lines, in which the gene is disrupted and which are lethal in the homozygous state; and/or antisense technology.

2. Over-express the proteins and look for chemicals which will bind to them and inhibit activity – mass screen.

3. Transform and express the plant protein in a microbe, having deleted the corresponding (functionally homologous) gene from the organism – (so that it is dependent on the plant protein for growth) then conduct a differential microbial screen using wild type vs transformed strains with a variety of chemical compounds.

Claims

1. A method of controlling the viability of a plant cell or cells by contacting the plant cell or cells with a substance which directly or indirectly up- or down-regulates a cell death pathway in the cell or cells, which pathway is activatable by depleting the concentration of NTP in the external environment available to the cell or cells whose viability is to be controlled.

2. A method according to claim 1, wherein the pathway is activatable by depleting the concentration of ATP in the external environment available to the cell or cells.

3. A method of killing a plant cell or plant cells according to claim 1 or 2, by activating a cell death pathway, which pathway is activatable by depletion of extracellular NTP, especially ATP, available to the cell for utilization.

4. A method according to claim 3, wherein the plant cell or cells are present in a plant and performance of the method results in death of the plant or a part thereof.

5. A method according to claim 3 or 4, wherein the cell death pathway is activated by depletion of extracellular ATP available to the cell for utilization.

6. A method according to any one of claims 3, 4 or 5, comprising the step of bringing an active agent into contact with the extracellular environment of the cell or cells to be killed, wherein the agent hydrolyses extracellular NTP (especially ATP) and/or renders extracellular NTP (especially ATP) non-available to the plant cell or cells.

7. A method according to any one of the preceding claims, comprising the use of an NTP (especially an ATP) analogue.

8. A method according to claim 7, wherein the NTP analogue comprises a phosphate group which is substantially non-hydrolysable by the plant cellular derived NTPase (especially ATPase) enzymes.

9. A method according to any one of the preceding claims, comprising the use of AMP-PCP.

10. A composition for controlling the viability of a plant cell or plant cells, the composition comprising an active agent which, directly or indirectly, up- or down-regulates in the cell or cells, a cell death pathway, which pathway is activatable by depletion of extracellular NTP (especially ATP) available for utilization by the cell or cells.

11. A composition according to claim 10, wherein the active agent is a substance which activates the cell death pathway.

12. A composition according to claim 10 or 11, for use in the method of any one of claims 1-9.

13. A composition according to claim 10, 11 or 12, further comprising one or more components of conventional herbicidal compositions, selected from the group consisting of surfactants and penetration enhancers.

14. A method of controlling the viability of a plant cell or cells substantially as hereinbefore described.

15. A composition for killing a plant cell or cells substantially as hereinbefore described.

16. A method of preserving the viability of a plant cell or cells exposed to viability-threatening extracellular NTP depletion, the method comprising the step of

administering a viability-preserving substance which has the effect, directly or indirectly, of increasing the extracellular NTP (especially ATP) concentration or otherwise inhibiting the cell death pathway which is activatable by depletion of extracellular NTP (especially ATP) available to the cells.

17. A method according to claim 16, wherein the viability-preserving substance comprises a nucleotide sequence and/or the polypeptide(s) encoded thereby, the nucleotide sequence encoding a polypeptide which, when expressed in the plant cell, has the effect of tending to increase the extracellular concentration of NTP (especially ATP) so as to preserve the viability of the cell.

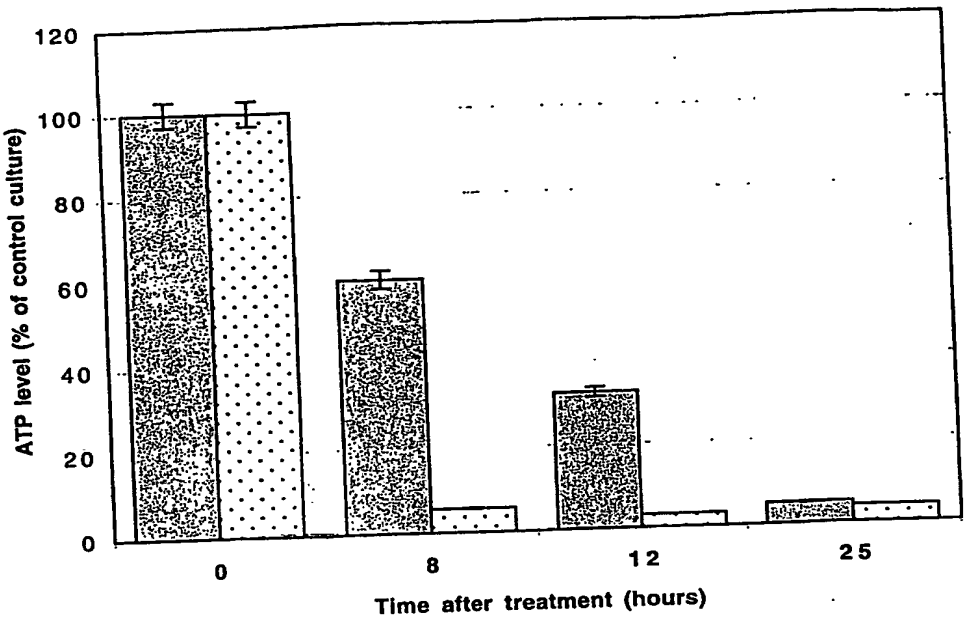
18. A method according to claim 17, wherein the nucleotide seuqence is expressed constitutively in the plant cell.

19. A method according to claim 17, wherein the nucleotide sequence is expressed in the plant cell in response to a viability-threatening event (e.g. attack by a pathogen).

ABSTRACT

Title: Improvements in or Relating to Plant Viability

Disclosed is a method of killing a plant cell or plant cells by activating a cell death pathway, which pathway is activatable by depletion of extracellular NTP, especially ATP, available to the cell for hydrolysis by cellular NTPase (especially ATPase) enzymes.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80
60
40
20
0
ATP level (% of control culture)
0
8
12
25
Time after treatment (hours)

Figure 1

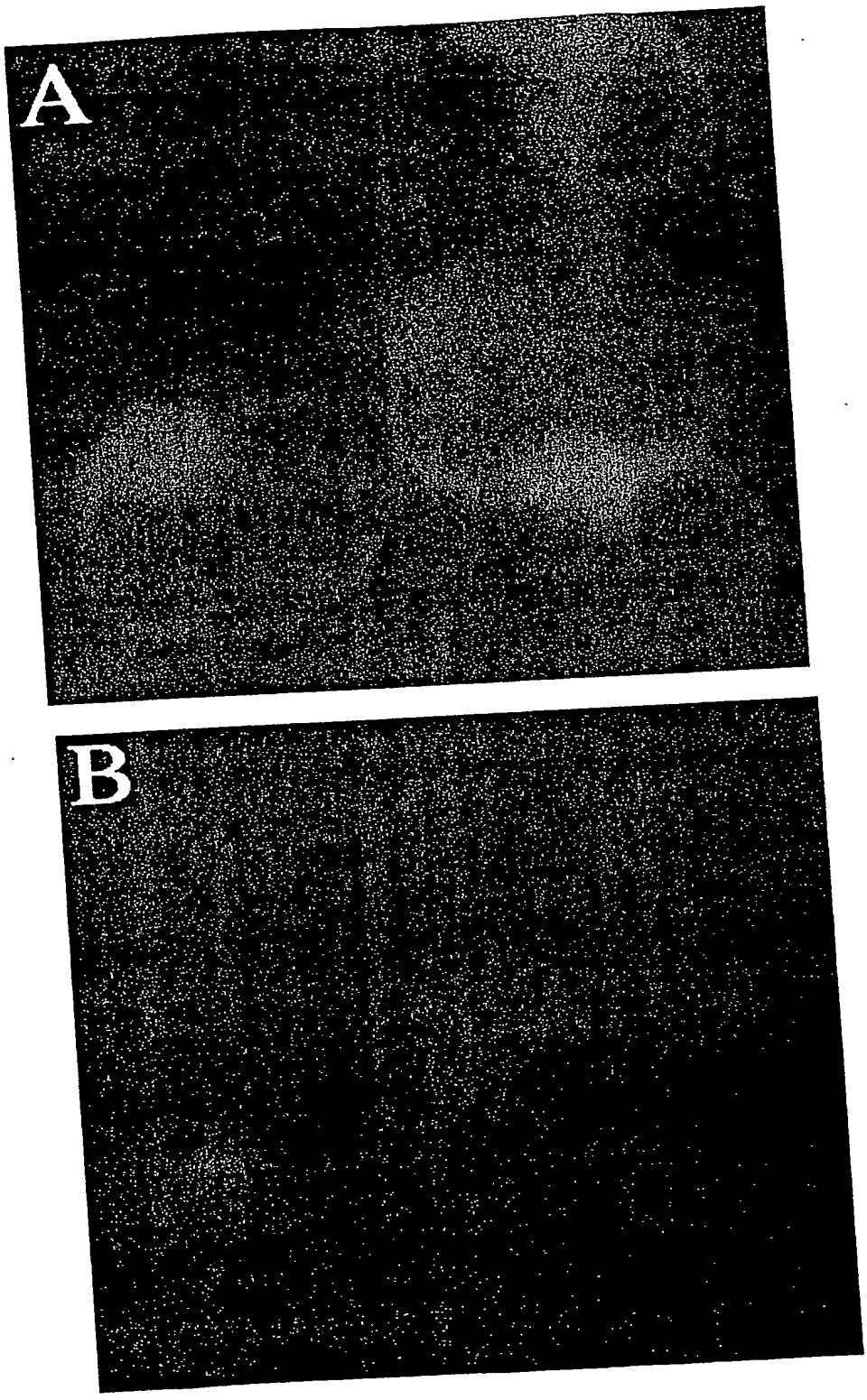
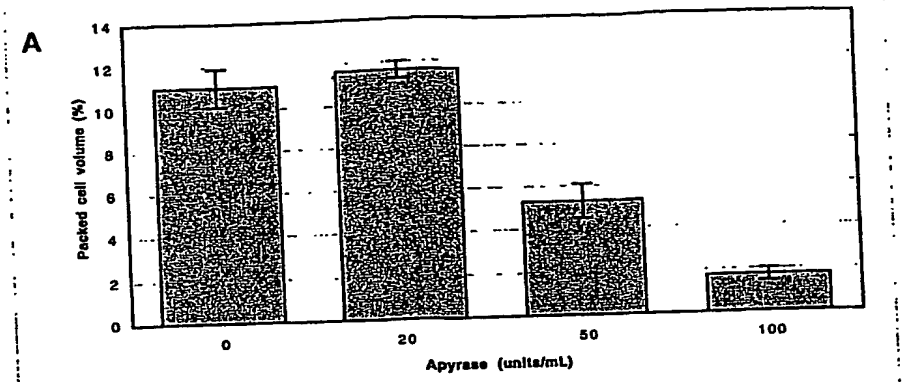


Figure 2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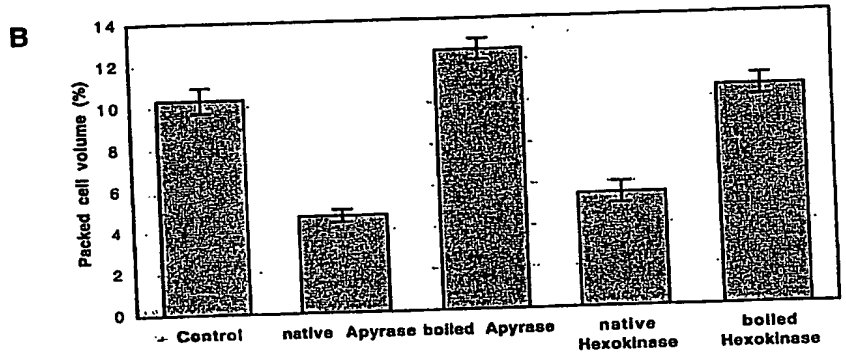
14
0
20
50
100
Apyrase (units/mL)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Packed cell volume (%)
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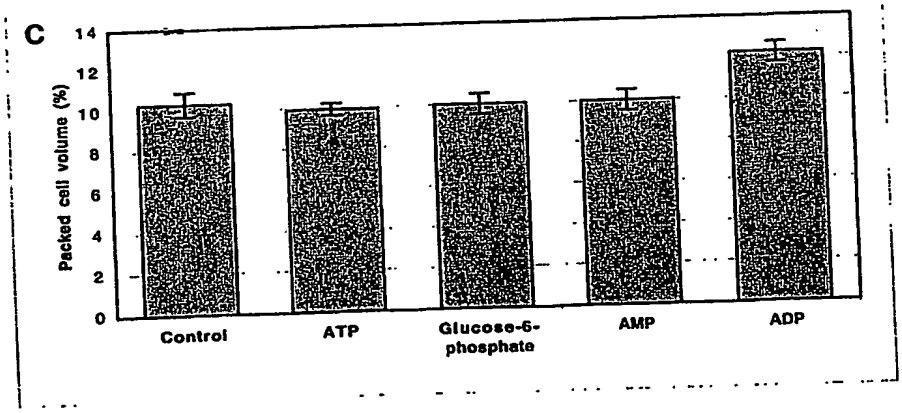
4
6
8
10
12
14
Control
native Apyrase
boiled Apyrase
native Hexokinase
boiled Hexokinase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Packed cell volume (%)
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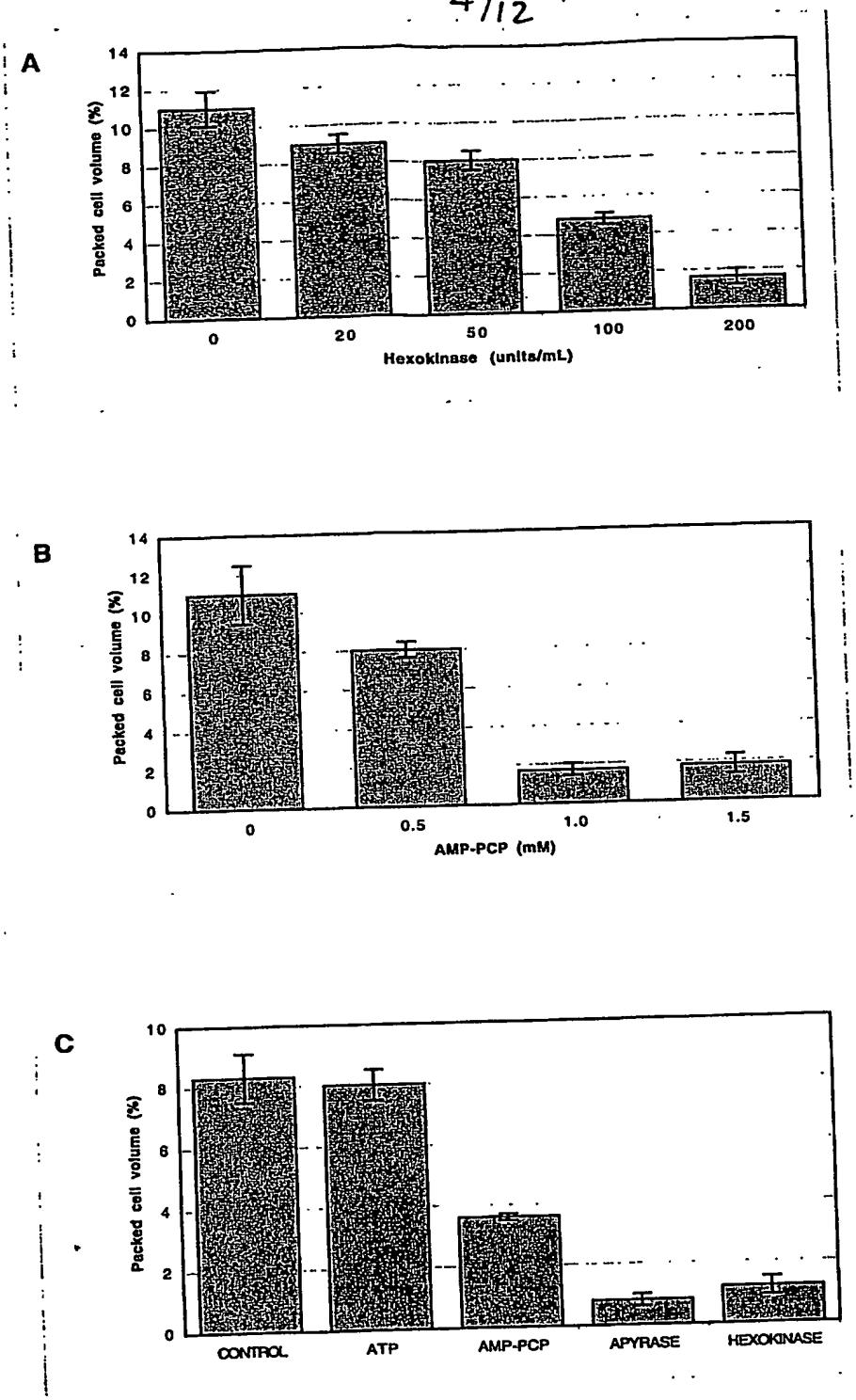
6
8
10
12
14
Control
ATP
Glucose-6-phosphate
AMP
ADP

Figure 3


A
Packed cell volume (%)
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2
4
6
8
10
12
14
0
20
50
100
200
Hexokinase (units/mL)
B
Packed cell volume (%)
0
2
4
6
8
10
12
14
0
0.5
1.0
1.5
AMP-PCP (mM)
C
Packed cell volume (%)
0
2
4
6
8
10
CONTROL
ATP
AMP-PCP
APYRASE
HEXOKINASE

Figure 4

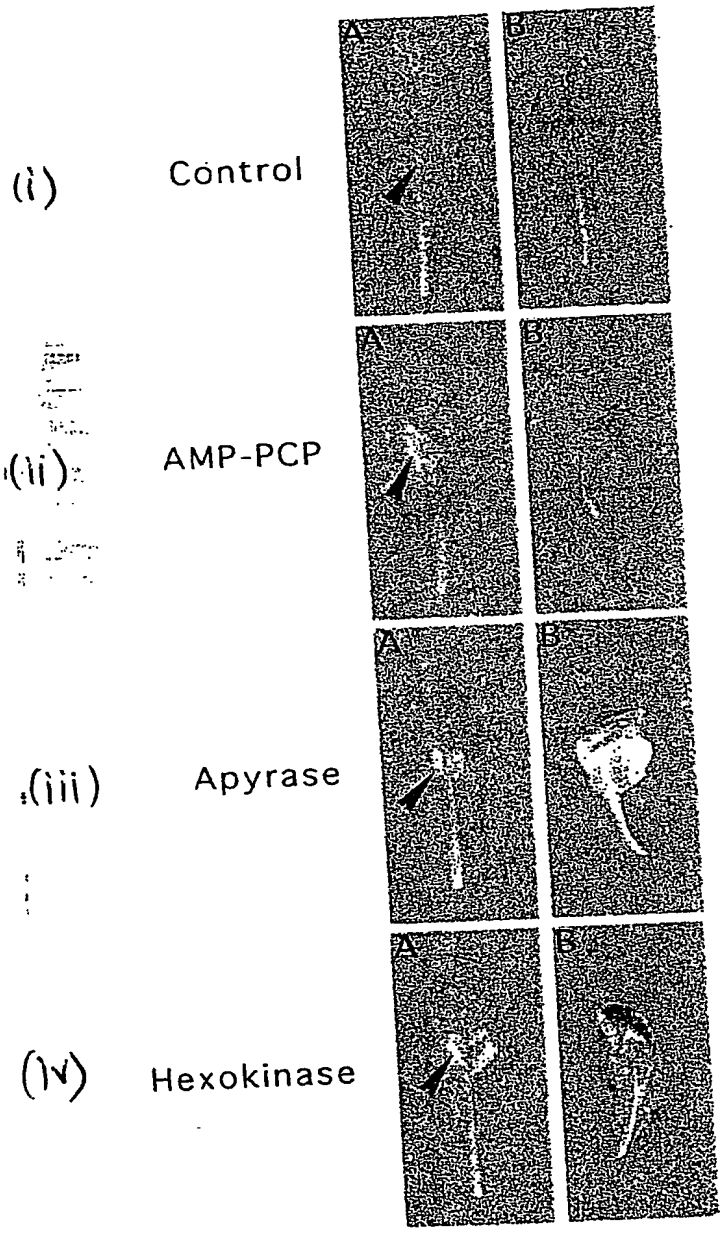


Figure 5

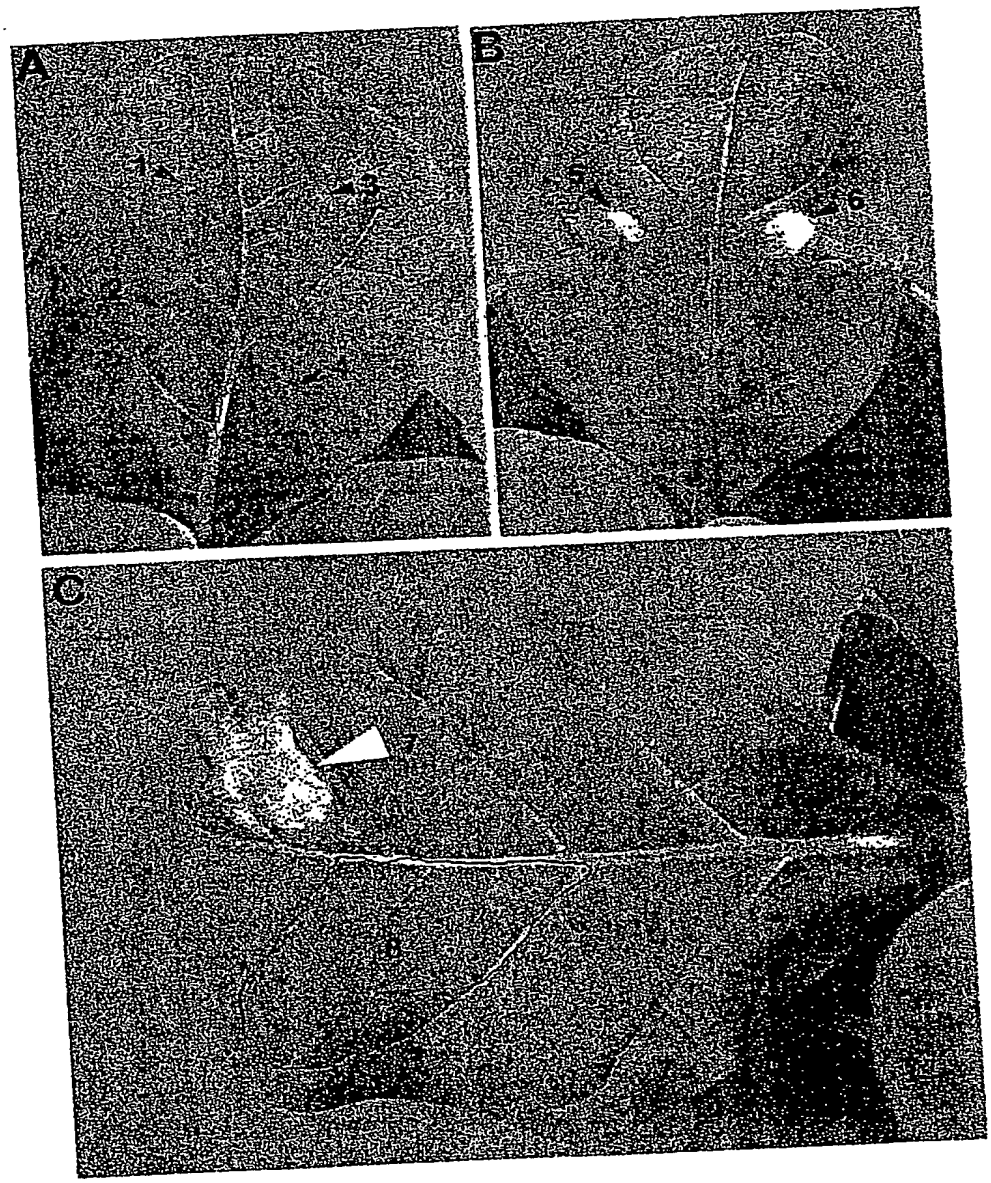


Figure 6

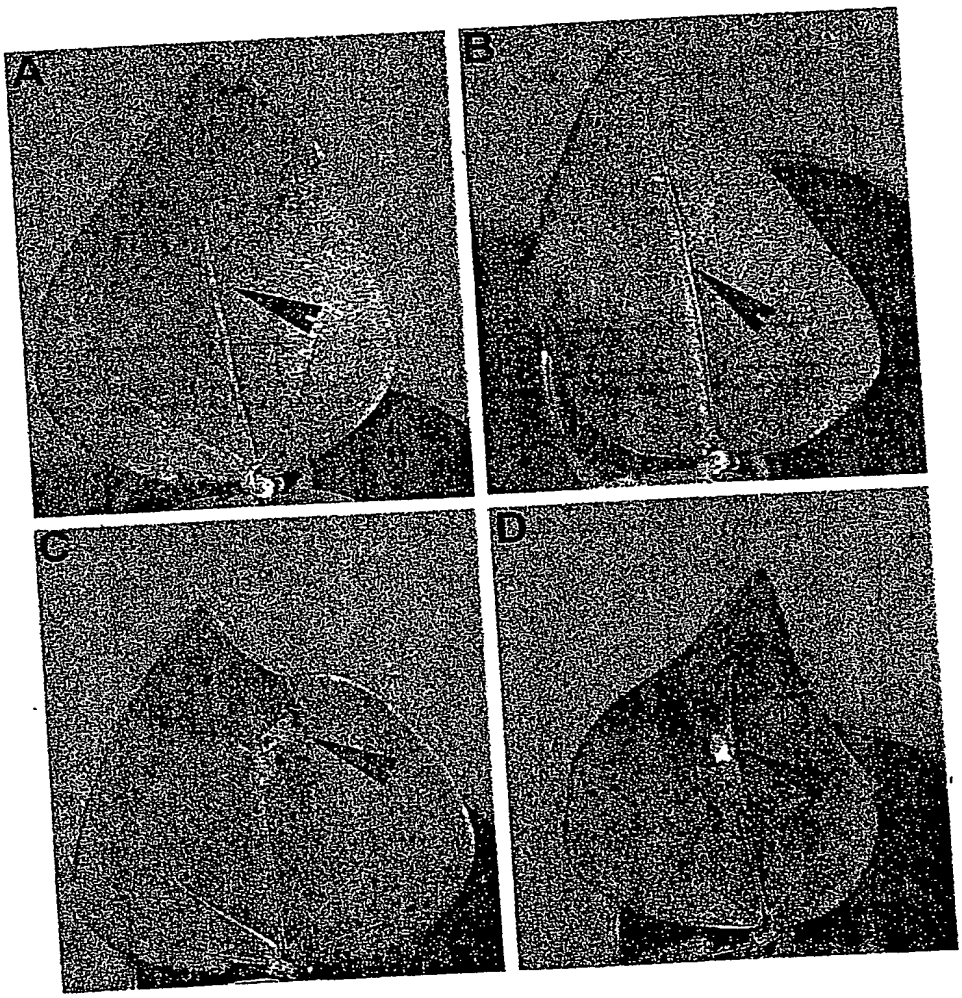


Figure 7

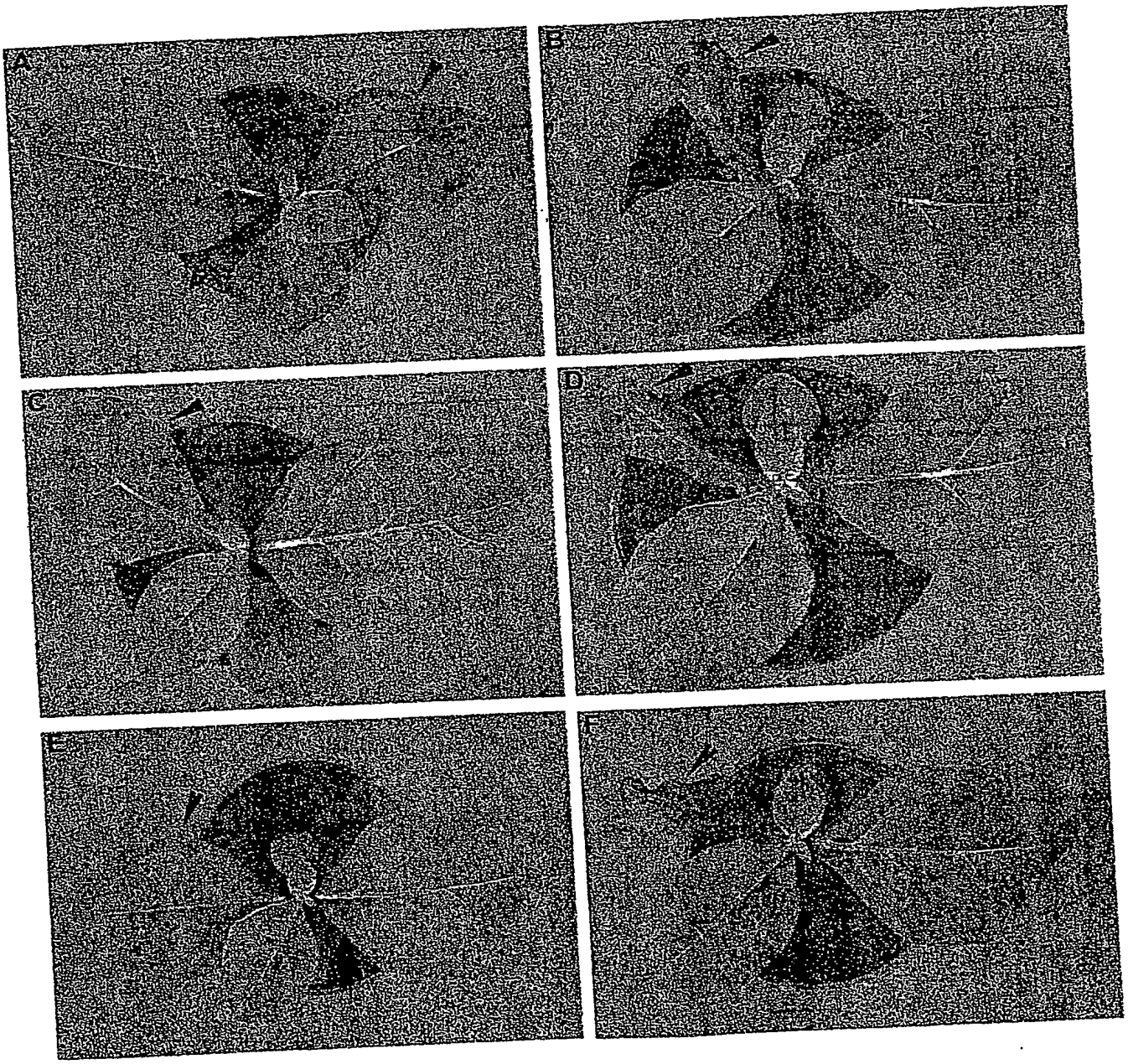


Figure 8

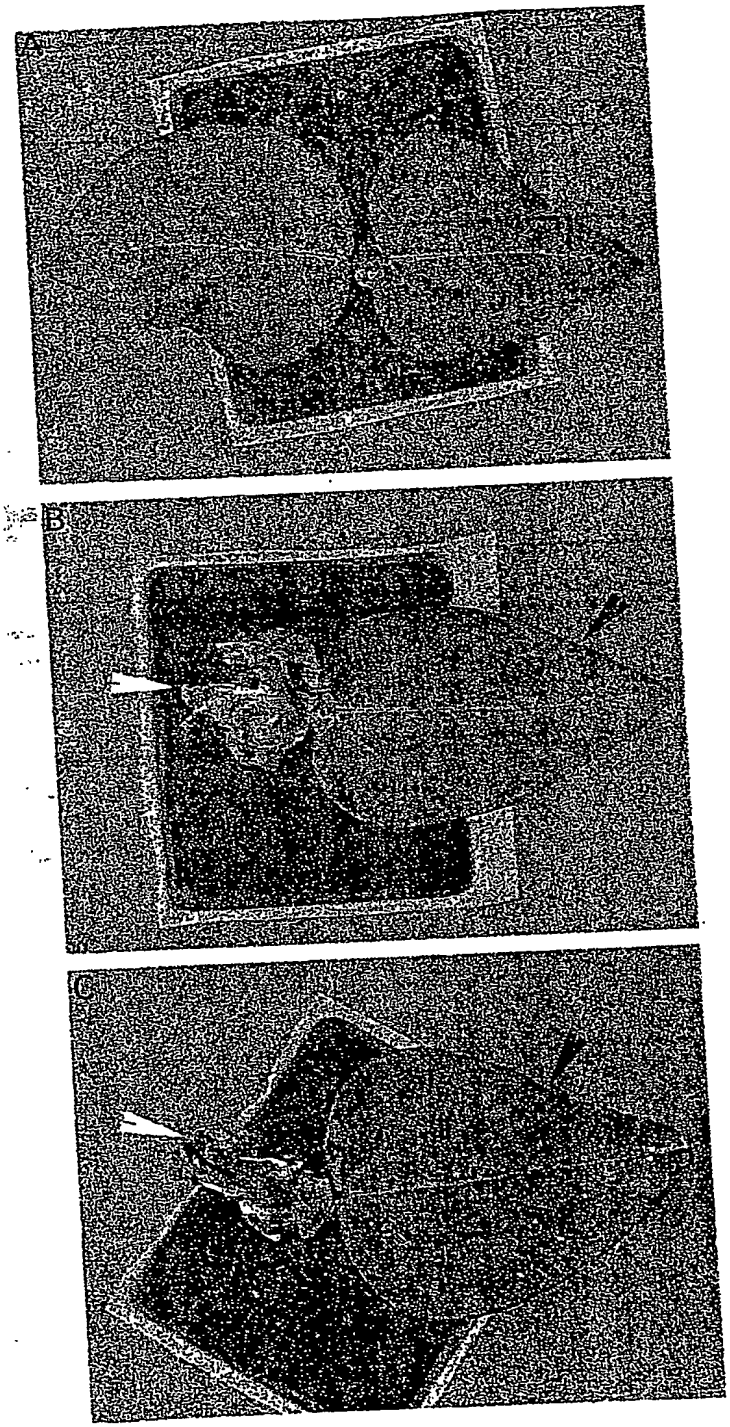
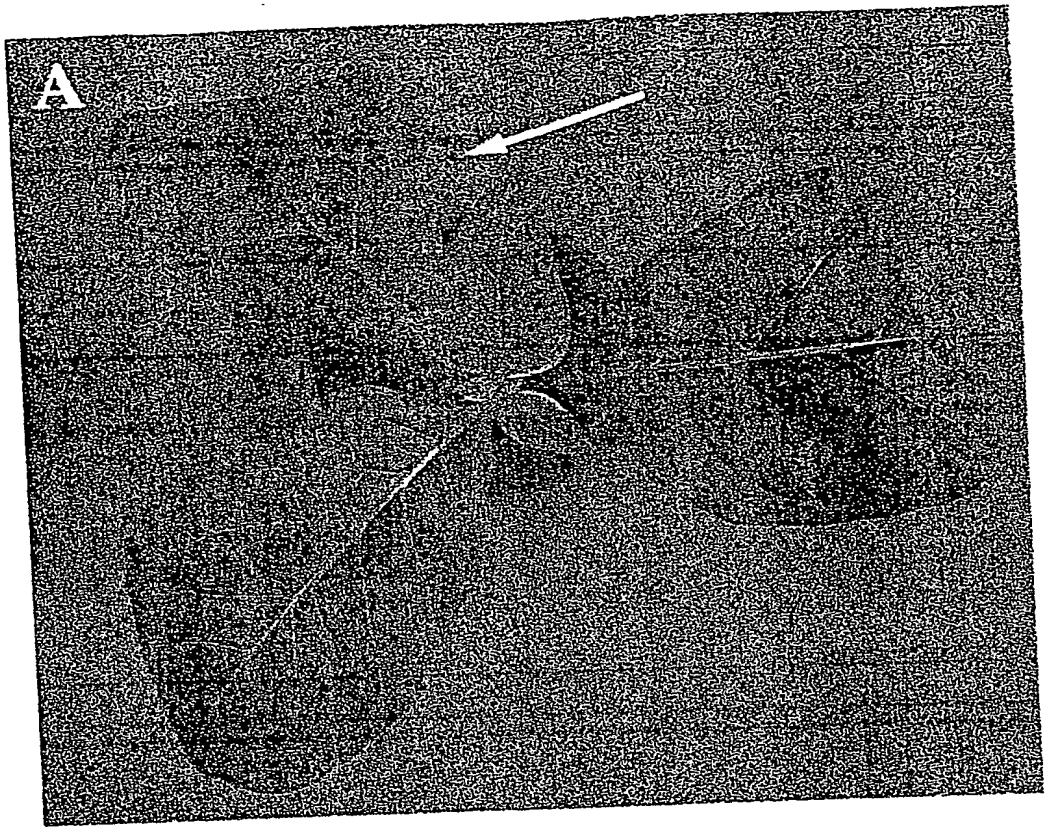


Figure 9



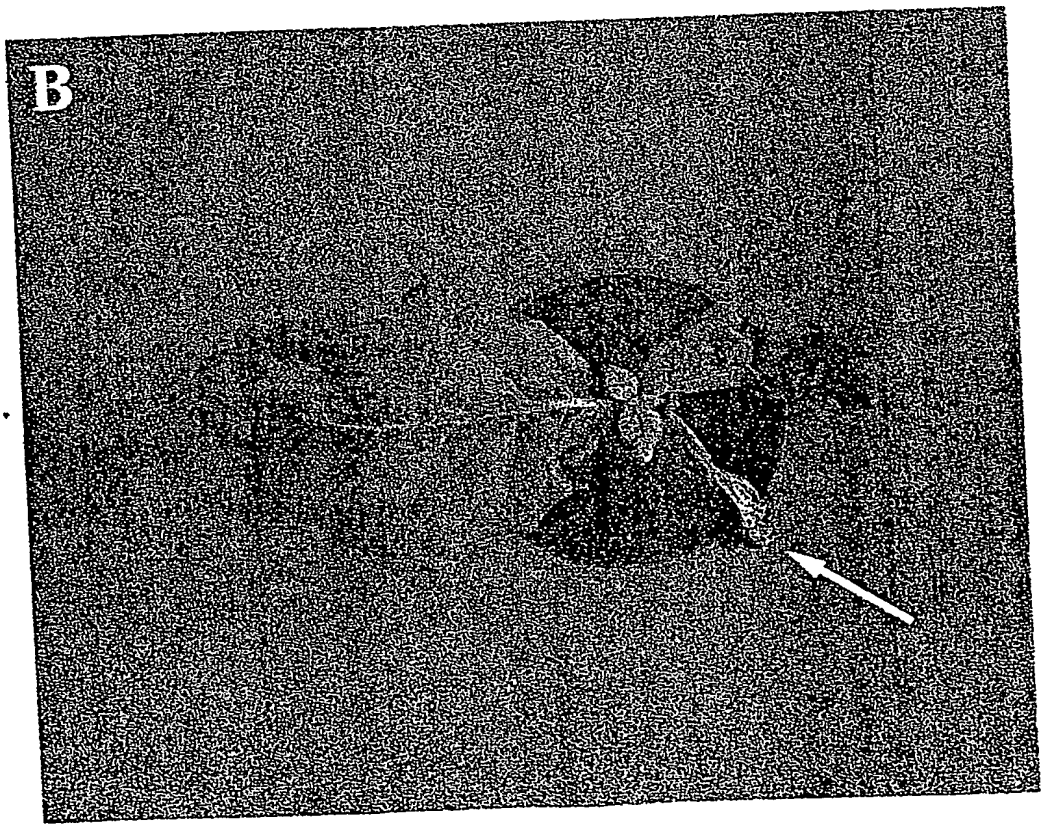


Figure 10

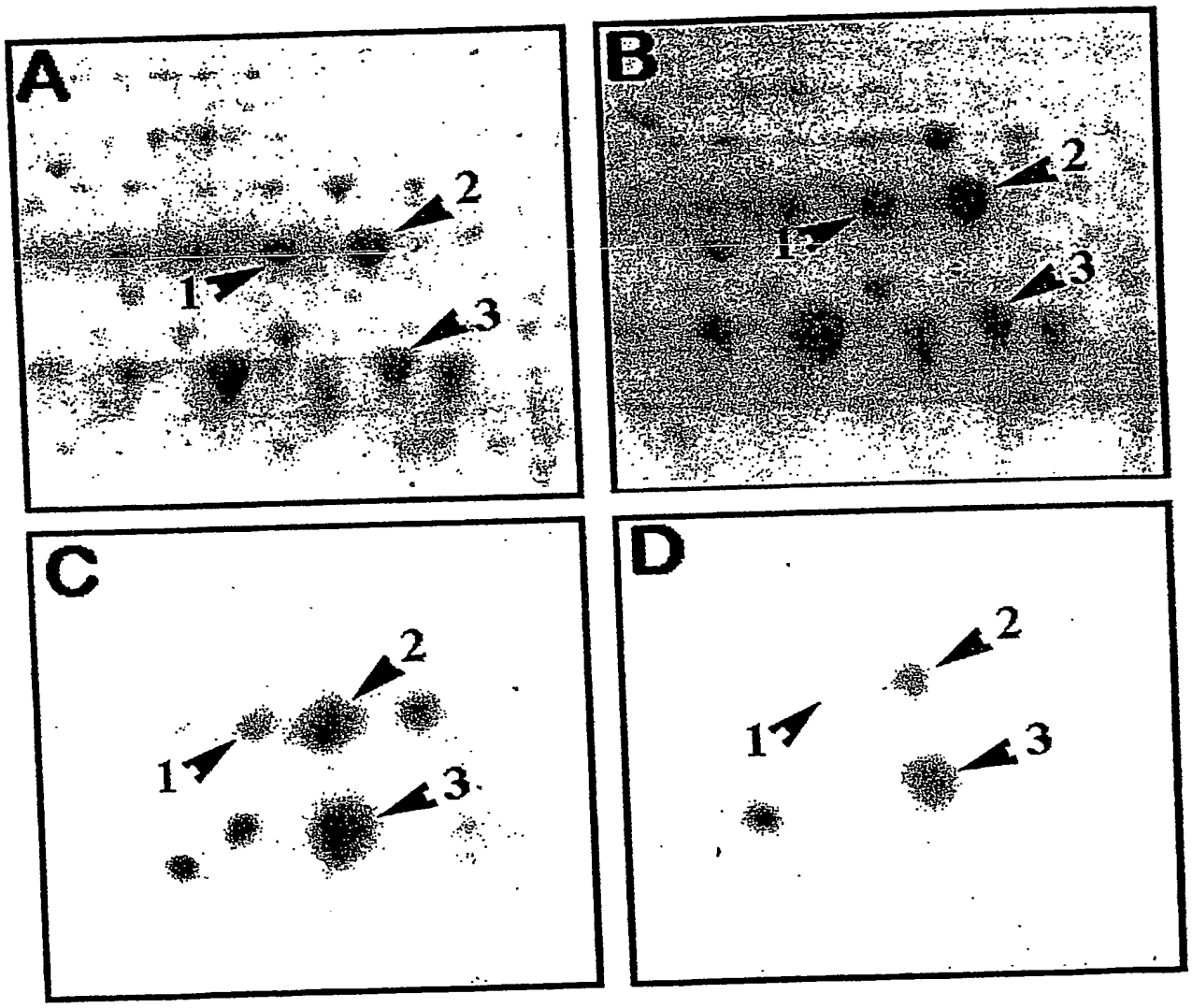


Figure 11

Fig. 12A

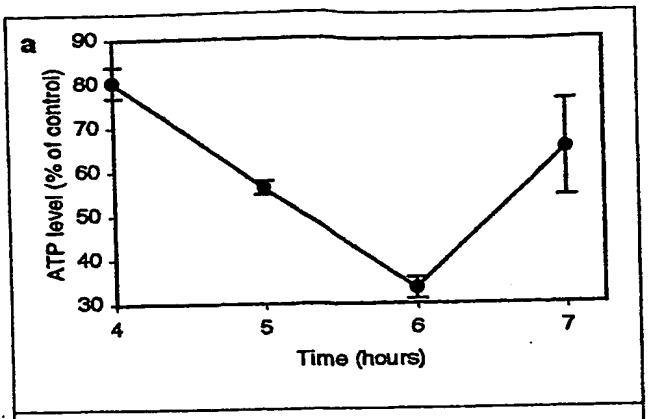


Fig. 12B